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Reversion of the Antichlamydial Effect of Tumor Necrosis Factor by Tryptophan and Antibodies to Beta Interferon†

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Human recombinant tumor necrosis factor-α (TNF-α) inhibited the growth of *Chlamydia trachomatis* (L_2/434/Bu) in HEp-2 cells. The effect was synergistic with that of gamma interferon (IFN-γ). TNF-induced resistance to chlamydiae could be blocked with cycloheximide, suggesting that it involves the function of some induced proteins. Tryptophan degradation was enhanced in the TNF-treated cells and was much further increased when the cells were treated with both TNF and IFN-γ at concentrations at which IFN-γ by itself had very little effect. Antibodies to IFN-β blocked the augmentation of tryptophan degradation by TNF and decreased but did not fully eliminate the antichlamydial effect of TNF. Increased concentration of tryptophan in the growth medium (>100 μg/ml) resulted in reversion of the antichlamydial effect of TNF. This study suggests that the inhibition of chlamydial growth by TNF is mediated partly through an autocrine function of IFN-β which, in synergism with TNF, enhances the activity of a tryptophan-degrading enzyme(s) and partly by some other activities of TNF which can be blocked by tryptophan.

Tumor necrosis factor (TNF) was initially identified as a serum factor that could exert antitumor activity in mice injected with bacterial lipopolysaccharide (9). Later studies revealed that TNF has a mediator role in the defense against a wide variety of pathogens. It is induced by bacteria (18), viruses (1), and certain eucaryotic parasites (5) and elicits a plethora of protective mechanisms against these infectious agents (2, 10, 12, 14, 22, 26).

Recently, we found that human recombinant TNF-α (rTNF-α) inhibits the growth of the obligate intracellular gram-negative bacterium *Chlamydia trachomatis* in HEp-2 cells (22). A similar inhibitory effect was found to be induced by gamma interferon (IFN-γ) (20).

Chlamydiae parasitize the host cell for nutrients and energy. Their development can therefore be arrested by deprivation of certain essential metabolites, such as amino acids. The most pronounced effect of such inhibition is the conversion of the parasite from the replicative form, the reticulate body, which is metabolically active, to the infective form, the elementary body (3, 4, 13). In certain human cells, IFN-γ treatment causes a decrease in tryptophan concentration, resulting from increased activity of the enzyme indoleamine 2,3-dioxygenase (IDOase) (16). In such cells, the IFN-induced cycle arrest of chlamydiae can be released by increasing the concentration of tryptophan in the cell growth medium (7, 19). Increase of tryptophan was also found to result in reversion of the inhibitory effect of IFN-γ on another intracellular protozoan, *Toxoplasma gondii* (15).

Some of the TNF effects are synergistic with that of IFN-γ (22, 24, 26). Furthermore, several studies have suggested that TNF-treated cells produce IFN-β, which mediates part of the antiviral effect of TNF (14, 17, 23).

In the present study, we investigated the extent of involvement of such induced IFN and of tryptophan degradation in the inhibition of *C. trachomatis* growth by TNF. Our findings reveal that IFN-β and enhanced degradation of tryptophan indeed contribute to the antichlamydial effect of TNF, but also suggest the involvement of some additional TNF-mediated activity(s) in this effect.

MATERIALS AND METHODS

***C. trachomatis* growth and purification.** The *C. trachomatis* serovar lymphogranuloma venereum (L_2/434/Bu) was grown in BGM cells in RPMI supplemented with 5% fetal calf serum (FCS), 1% glucose, 100 μg of streptomycin per ml, 10 μg of glutamine per ml, 10 μg of Fungizone per ml, and 1 μg of cycloheximide per ml. The chlamydiae were harvested and purified as previously described (20).

Cells. HEp-2 cells, originating from human carcinoma of the larynx (Flow Laboratories, cell line 03-108) were grown in minimal essential medium (MEM) containing the specified concentration of tryptophan (Biological Industries, Bet Haemek, Israel), 10% FCS, glutamine, and antibiotics.

TNF. Human rTNF-α (6×10^7 U/mg of protein; produced by Genentech Co., San Francisco, Calif.), containing less than 0.125 endotoxin units per ml (as determined by the *Limulus* amoebocyte lysate assay) was kindly provided by G. Adolf, Boehringer Institute, Vienna, Austria.

IFN. Human recombinant IFN-γ and IFN-β were a gift from Inter-Yeda Inc., Rehovot, Israel.

Abs. Rabbit polyclonal antibodies (Abs) to human IFN-β were a gift of A. Zilberstein and M. Revel, The Weizmann Institute, Rehovot. The mouse monoclonal Abs to IFN-β were obtained from Inter-Yeda Inc., Rehovot, Israel. Rabbit polyclonal Abs to TNF were produced in one of our laboratories (D.W.). Incubation of the cytokines with Abs before application on cells was carried out for 0.5 h at room temperature. Monoclonal antibodies to IFN-β were used at 0.4 mg of protein per ml, and polyclonal antiserum to TNF was used at a dilution of 1:100.

One-step growth-yield assay for chlamydiae. HEp-2 cells were kept for 24 h in medium which contained vancomycin and gentamicin instead of penicillin. They were then seeded in 96-well microtiter plates at 3×10^4 cells per well. The following day, the medium was replaced with growth me-

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TABLE 1. Suppression of TNF antichlamydial effect with cycloheximide and antibodies to IFN-β[a]

Treatment	Chlamydial yield (IFU/ml)		
	No treatment	Cycloheximide (10 μg/ml)	Monoclonal Abs to IFN-β
None	3.5 (±1.3) × 10^7	2.3 (±0.3) × 10^6	2.7 (±0.3) × 10^7
TNF (250 ng/ml)	2.4 (±0.2) × 10^2	4.3 (±1) × 10^5	2 (±0.4) × 10^5
IFN-γ (2 U/ml)	1.5 (±0.3) × 10^7	9.9 (±1) × 10^5	2.5 (±0.6) × 10^7
TNF (250 ng/ml) and IFN-γ (2 U/ml)	26 ± 46	1.5 (±0.2) × 10^6	2 (±0.4) × 10^4
IFN-β (500 U/ml)	3.7 (±0.3) × 10^4		1.6 (±0.3) × 10^7
IFN-β (5,000 U/ml)	3.1 (±0.6) × 10^4		2.1 (±0.1) × 10^5

[a] The results represent one of three similar experiments. Similar results were obtained with the use of polyclonal Abs to IFN-β.

dium containing 2% FCS and TNF at various concentrations. Cells were incubated further at 37°C for 24 h, and purified *C. trachomatis*, diluted in *C. trachomatis* growth medium (MEM supplemented with 5% FCS, glutamine [10 μg/ml], vancomycin [0.1 mg/ml], gentamicin [16 μg/ml], and 1% glucose), was applied at a multiplicity of infection of 1. After 1 h of adsorption at 37°C, the inoculum fluid was removed, and cells were rinsed once and further incubated with 100 μl of *C. trachomatis* growth medium per ml. After an additional 48 h of incubation, the infected cells were scraped into the medium. Triplicate wells were pooled, and the samples were frozen at −70°C. The yield of chlamydiae, expressed as inclusion-forming units (IFU) per milliliter, was determined by titrating the samples on HEp-2 cells in the presence of *C. trachomatis* growth medium containing cycloheximide as described previously (20). After 48 h, the cells were fixed for 10 min in absolute ethanol, and the immunoperoxidase assay was performed on the plate.

For the 48-h tryptophan pulse experiments (see Table 3), the procedure was as follows. After 48 h, confluent HEp-2 cells were infected with *C. trachomatis* for 1 h, and then the inoculum was removed and the cells were left untreated, treated with TNF (500 ng/ml), or treated with IFN-γ (100 U/ml) in the presence of MEM (without phenol red) that was supplemented with 2% FCS, glutamine, antibiotics, and [^{3}H]tryptophan (5 μCi/ml). The cells were incubated for 48 h at 37°C, and then the yield of chlamydiae was determined in a pool of triplicate wells. Samples were also taken for evaluation of tryptophan catabolism.

Determination of tryptophan degradation. Tryptophan degradation in HEp-2 cells was determined by using a modification of the procedure described by Byrne et al. (8). Briefly, HEp-2 cells were seeded in 96-well microplates at 3 × 10^4 cells per well. The following day, tested cytokines (TNF, IFN-γ, and IFN-β) were applied to the cells, in triplicate, in the presence of growth medium containing 2% FCS. At various times after treatment (usually 48 h), the medium was removed and cells were pulse labeled for 4 h by applying 100 μl of Hanks's balanced salt solution, containing 0.1 μCi of L-[G-^{3}H]tryptophan (Amersham, Buckinghamshire, England), and 1 μg of nonlabeled tryptophan per well. In parallel, a sample of the solution containing the labeled tryptophan was incubated at 37°C without cells for determining nonspecific degradation of tryptophan. After 4 h of incubation, the medium was collected, clarified by centrifugation, and frozen at −70°C until analysis. Alternatively, to assess the extent of the contribution of tryptophan degradation to the antichlamydial effect of TNF, we incubated chlamydia-infected cells for 48 h with TNF and ^{3}H-labeled tryptophan (5 μCi/ml). The amounts of tryptophan and its catabolites *N*-formylkynurenine and kynurenine in the growth medium were estimated by reversed-phase high-pressure liquid chromatography analysis as described by Yong and Lau (27). Samples (50 μl) were injected into an Ultrapac column (Lichrosorb RP; 18.5 μm) and eluted in 10% methanol at a flow rate of 0.8 ml/min.

Elution times of tryptophan and its catabolites was monitored by determining UV absorbance at 254 nm (for kynurenine and *N*-formylkynurenine) and 280 nm (for tryptophan). Fractions were collected at 30-s intervals, and their content of radioactivity was determined by scintillation spectrometry. The extent of catabolism of tryptophan in the medium was calculated by using the following equation: percent specific catabolism = $[(cpm_{test} - cpm_{spontaneous})/(cpm_{total} - cpm_{spontaneous})] \times 100$, where cpm_{test} represents the counts present in the tryptophan catabolite fractions, $cpm_{spontaneous}$ is the counts in the same fractions after incubation in the absence of cells, and cpm_{total} is the sum of cpm from tryptophan and tryptophan catabolite fractions. In the 48-h pulse experiments (see Table 3), 48% ± 15% of the counts that were applied could be recovered in the growth medium of both TNF-treated and untreated control cells. The decrease in radioactivity found in the tryptophan peak was calculated as follows: D (% specific degradation) = [(% test − % spontaneous)/(100 − % spontaneous)] × 100, where % test represents the percentage of total radioactivity present in the tryptophan catabolite fraction and % spontaneous represents the percentage of counts in the same fractions after incubation of medium for 48 h in the absence of cells.

Electron microscopy. Samples were prepared for electron microscopy according to the method of Biberfeld (6). HEp-2 infected cells were fixed for 60 min with 2% glutaraldehyde in cacodylate buffer and washed twice with cacodylate buffer. Postfixation was done with 1% OsO_4 for 60 min, followed by dehydration in alcohol and three washes with propylene oxide. Embedding was done in Araldite 502. The blocks were sectioned and stained with uranyl acetate and lead citrate. Electron micrographs of the thin sections were taken with a Phillips 201C transmission electron microscope.

RESULTS

Inhibition of the antichlamydial effect of TNF by cycloheximide and Abs to IFN-β. To explore the involvement of induced proteins, and more specifically induced IFN-β, in the inhibition of chlamydial growth by TNF, we tested the effects of the protein synthesis inhibitor cycloheximide and Abs to IFN-β on this phenomenon. As shown in Table 1, the antichlamydial effect of TNF could be almost fully blocked with cycloheximide (10 μg/ml). The same extent of inhibition was observed when TNF and cycloheximide were applied to the cells either 24 h prior to infection (Table 1) or immediately after infection (data not shown). A marked decrease in

the extent of inhibition was also observed when the cells were treated with either monoclonal or polyclonal Abs to IFN-β. This reversion, however, was only partial. A decrease of at least 100-fold in chlamydial yield was still observed when the HEp-2 cells were treated with TNF in the presence of Abs to IFN-β at concentrations sufficient to neutralize as much as 500 U of IFN-β per ml (Table 1). On the other hand, Abs to TNF fully blocked the antichlamydial effect of this cytokine (data not shown). Antibodies to IFN-β also effectively decreased the inhibition of *C. trachomatis* growth by TNF combined with IFN-γ (2 U/ml) (Table 1).

Suppression of the antichlamydial effect of TNF by tryptophan. In certain cells, including HEp-2 cells, the antichlamydial effect of IFN-γ can be blocked by exposing the IFN-treated cells to increased tryptophan concentrations (8, 19). As shown in Fig. 1 and 2, the inhibitory effect of TNF on the growth of *C. trachomatis* in HEp-2 cells was also effectively suppressed by increasing the concentration of tryptophan in the cell growth medium. Partial reversion of the antichlamydial effect could be observed with as little as 50 μg of tryptophan per ml. At a tryptophan concentration of 100 μg/ml, TNF caused a decrease in chlamydial yield of less than 10-fold, as opposed to as much as a 1,000-fold in the presence of the normal tryptophan concentration (10 μg/ml). At higher concentrations (500 μg/ml), tryptophan by itself had some inhibitory effect (Fig. 2). Tryptophan also reversed the more effective inhibition of chlamydial growth observed after combined treatment with TNF and IFN-γ (2 U/ml) (Fig. 1 and 2). Even at high concentrations of tryptophan, a slight antichlamydial effect of TNF was still observed; it was fully abolished when the cells were treated with a high tryptophan concentration together with Abs to IFN-β (Fig. 3).

In ultrastructural analysis by electron microscopy, chlamydia-infected HEp-2 cells treated with TNF were found to contain relatively smaller inclusion bodies which enclosed enlarged reticulate bodies and other irregular forms of the chlamydial development cycle (Fig. 4A). In contrast, in the presence of an elevated tryptophan concentration, the TNF-treated cells contained typical large inclusion bodies (Fig. 4B), which resembled those observed in infected cells not treated with TNF (Fig. 4C).

Enhancement of tryptophan degradation by TNF and its reversal with Abs to IFN-β. After treatment with TNF or IFN-γ, the HEp-2 cells exhibited enhanced degradation of tryptophan. The maximal effect of TNF was lower than that of IFN-γ and was significantly potentiated by applying IFN-γ simultaneously with TNF at concentrations at which IFN by itself had no apparent effect (Table 2). Determination of the extent of degradation of tryptophan after prolonged incubation under the conditions of the test for the antichlamydial function of TNF revealed no correlation between the effectiveness of that function and the extent of tryptophan degradation in the growth medium. In some of the experiments, tryptophan was almost fully depleted in medium from TNF-treated cells. However, in others in which the antichlamydial effect was just as marked, only part of the tryptophan was found to be degraded (Table 3). The effect of TNF on tryptophan degradation was almost fully abolished when the cells were treated with both TNF and Abs to IFN-β (Table 2), and yet, as mentioned above, cells treated with TNF in the presence of the Abs exhibited an antichlamydial effect, although to a reduced extent (Table 3).

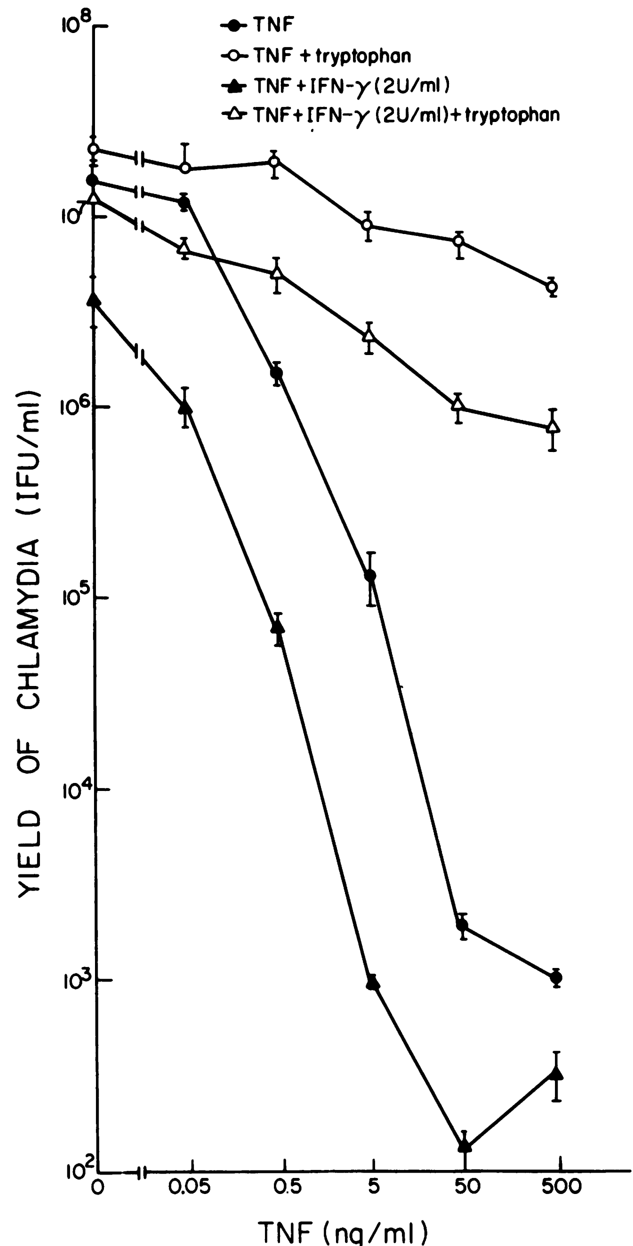

FIG. 1. Dose-response curve of the effect of TNF-α on *C. trachomatis* growth in either the presence or the absence of IFN-γ and the suppression of this effect by increased tryptophan concentration. The HEp-2 cells were treated 24 h before infection with TNF in either the presence or absence of IFN-γ (2 U/ml). As indicated, a portion of the cultures were incubated in the presence of an increased concentration of tryptophan (200 μg/ml) following chlamydial absorbance. Chlamydial yield was determined 48 h after infection.

DISCUSSION

This study provides initial information concerning the mechanism of the inhibition of *C. trachomatis* by TNF. The inhibitory effect is suppressed when the synthesis of cellular proteins is blocked with cycloheximide, as well as by elevation of the tryptophan concentration in the cell growth medium, and by application of specific Abs to IFN-β.

These findings suggest that the effect of TNF on chlamydial growth involves the function of induced proteins and furthermore that these proteins include IFN-β and an enzyme(s) which enhances degradation of tryptophan, possibly IDOase.

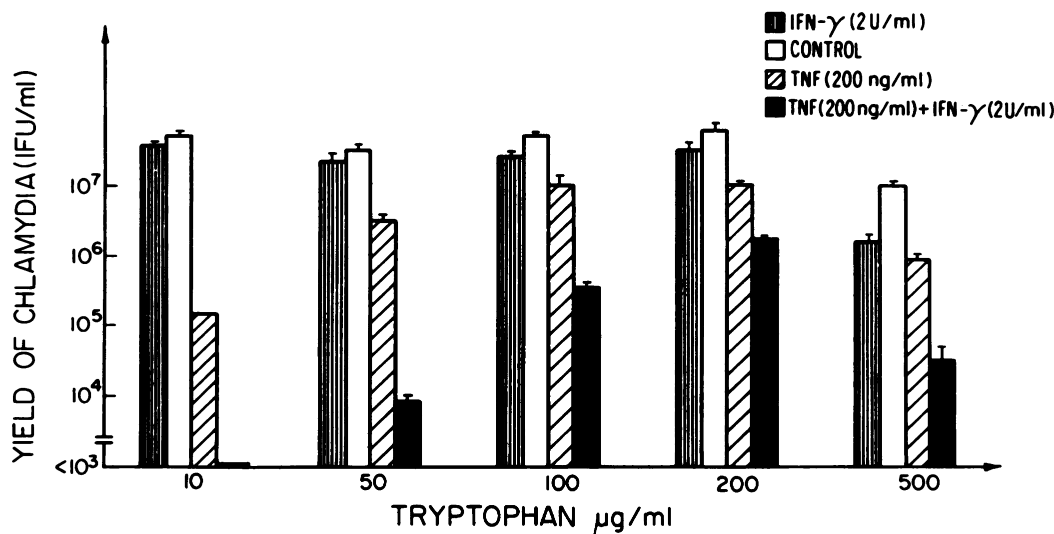

FIG. 2. Effect of tryptophan concentration on *C. trachomatis* inhibition by TNF. HEp-2 cells were treated 24 h before infection with rTNF-α (200 ng/ml) in either the presence or the absence of IFN-γ (2 U/ml). Medium applied after infection contained tryptophan either at the standard (10 μg/ml) or at increased concentrations (50, 100, 200, and 500 μg/ml). Chlamydial yield was determined 48 h after infection. The results shown are from one of four similar experiments.

Tryptophan is an essential amino acid for chlamydiae, and it has been suggested that a decrease in its intracellular pool, as a consequence of increased IDOase activity (16), underlies the IFN-γ-induced antichlamydial effect (7, 21). The fact that the antichlamydial effect of TNF can be suppressed by increasing the concentration of tryptophan in the cell growth medium (Fig. 1 and 2) suggests a similar role for the enhancement of tryptophan degradation by this cytokine (Tables 2 and 3). It should be noted, though, that the expression of an antichlamydial effect which can be blocked by tryptophan appears to be only partly correlated with the ability of the cells to degrade this amino acid (Table 3). One possibility is that the extent of decrease in extracellular tryptophan following treatment with TNF or IFN is just a partial reflection of a much more sustained decrease inside the cells. Alternatively, these findings may imply that tryptophan counteracts the antichlamydial effect of TNF not only by overcoming the degradation of tryptophan, which is an essential amino acid for *C. trachomatis* growth, but also in other ways. Recently we have shown that simultaneous treatment of HEp-2 cells with *C. trachomatis* and TNF resulted in high production of prostaglandin E_2 (PGE_2),

TABLE 2. Effect of Abs to IFN-β and to TNF on induction of tryptophan catabolism by various cytokines[a]

Cytokine treatment	% Specific tryptophan catabolism per 4 × 10^4 cells during 4-h pulse		
	No treatment	Monoclonal Abs to IFN-β	Abs to TNF
None	1 ± 2	1.5 ± 2	3 ± 3
IFN-γ (2 U/ml)	1 ± 1	0	1.5 ± 1
TNF (500 ng/ml)	11 ± 2	0	0.5 ± 0.7
TNF (500 ng/ml) and IFN-γ (2 U/ml)	55 ± 23	0.3 ± 3	0
IFN-γ (200 U/ml)	92 ± 1	91 ± 1	92
IFN-β (500 U/ml)	3 ± 4	0.1 ± 1	0
IFN-β (5,000 U/ml)	9	0	ND[b]

[a] This table represents aggregate results of six experiments.
[b] ND, Not done.

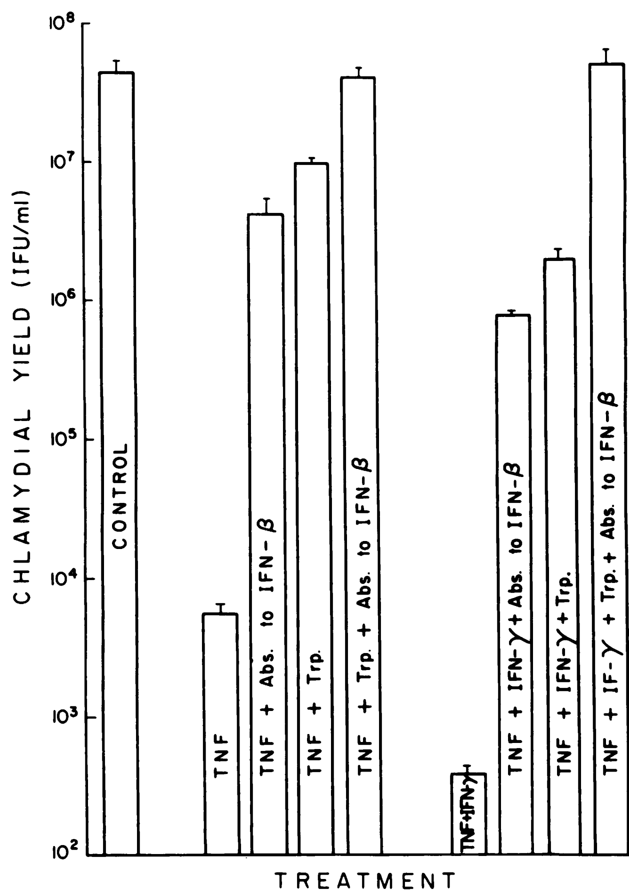

FIG. 3. Effect of combined treatment with tryptophan and antibodies to IFN-β on *C. trachomatis* inhibition by rTNF-α. HEp-2 cells were pretreated 24 h before infection with rTNF-α (500 ng/ml) either alone or with IFN-γ (2 U/ml). When indicated, monoclonal antibodies to IFN-β were applied at a concentration of 0.4 mg/ml, and tryptophan concentration in the medium was increased from 10 to 200 μg/ml. Chlamydial yield was determined 48 h after infection. The results shown are from one of three similar experiments.

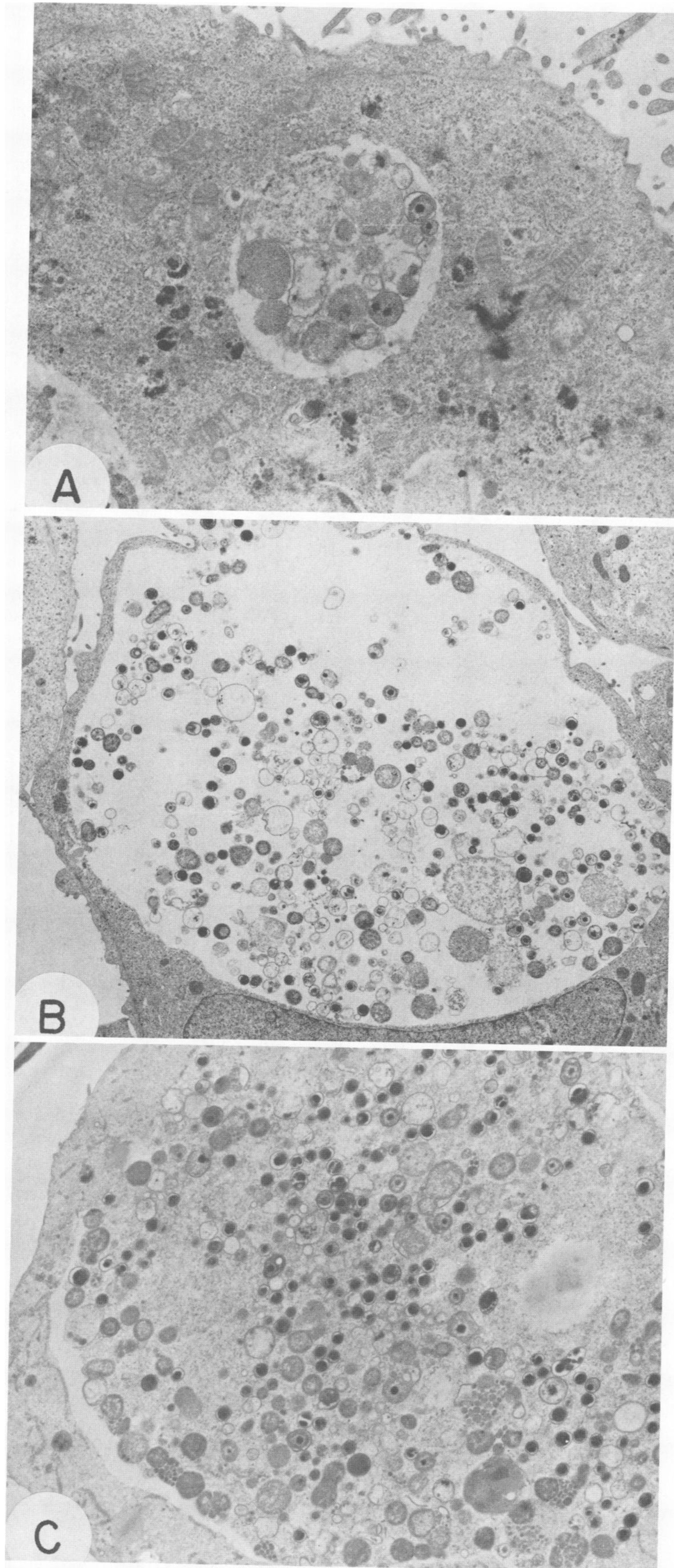

FIG. 4. Electron micrographs of *C. trachomatis* development in HEp-2 cells 48 h after infection. (A) Treatment with rTNF-α (200 ng/ml); tryptophan concentration in the medium, 10 μg/ml; magnification, ×7,600. (B) Treatment with rTNF-α (200 ng/ml) in the presence of increased tryptophan concentration (110 μg/ml); magnification, ×4,900. (C) Control, no TNF treatment; tryptophan concentration in the medium, 10 μg/ml; magnification, ×7,600.

TABLE 3. TNF Effects on chlamydial yield and tryptophan degradation during 48-h pulse

Treatment	Expt 1		Expt 2		Expt 3		Expt 4
	Chlamydial yield (IFU/ml)	D^a (%)	Chlamydial yield (IFU/ml)	D (%)	Chlamydial yield (IFU/ml)	D (%)	D (%)
None (control)	1.4×10^7	11.8	1.8×10^7	12.5	5×10^6	19	13.7
TNF	1.0×10^4	93	1.0×10^5	96	7×10^2	23.5	40
TNF + Abs to IFN-β			9.6×10^5	24			
IFN-γ	8.3×10^5	38	1.0×10^5	96	1×10^3	40	

[a] D, Percent specific degradation (see Materials and Methods).

which could be blocked by an elevated concentration (>100 μg/ml) of tryptophan (manuscript in preparation). Thus, tryptophan might inhibit the production of PGE_2, which has been shown by Ward and Salari (25) to affect the development of chlamydiae. Another possibility is that tryptophan may function as a free-radical scavenger and may thus protect the chlamydiae from the destructive effects of oxygen radicals induced by TNF. Further studies are required in order to clarify the mechanism by which tryptophan reverses the inhibition of *C. trachomatis* by TNF.

It has been suggested that the antiviral effects of TNF are, at least in part, mediated by IFN-β (14, 17, 23). The suppression of TNF-induced degradation of tryptophan and the partial reversion of the antichlamydial effect of TNF by antibodies to IFN-β (Tables 1 and 2) suggests that IFN also plays a mediator role in these effects. There are, however, several pieces of evidence which indicate that the generation of IFN-β might not fully account for the antichlamydial effect of TNF. First, the antichlamydial effect of TNF was only partly suppressed with antibodies to IFN-β, even at concentrations of antibodies which blocked the antichlamydial effect of as much as 500 U of IFN-β per ml (Table 1). Second, treating the HEp-2 cells even with high (5,000 U/ml) concentrations of IFN-β did not suppress the growth of chlamydiae as effectively as treatment with TNF (Fig. 1 and Table 1).

Though TNF has recently been shown to induce low amounts of mRNA for IFN-β in HEp-2 cells, secretion of IFN-β itself by cells has so far eluded detection and possibly occurs at very low levels (11). Recently, Reis et al. have shown that in inducing an antiviral state in FS-4 cells, TNF synergizes with very small amounts of IFN-β (≤0.2 U/ml), which might be produced spontaneously by cells even in the absence of TNF (L. F. L. Reis, T. H. Lee, Y. Zhang, J.-X. Lin, M. Kohase, T. Fujita, T. Taniguchi, and J. Vilcek, Proc. Annu. Meet. Int. Soc. IFN Res. Kyoto, Japan, 1988, in press). We also found that we could not potentiate further the antichlamydial effect of TNF by addition of exogenous IFN-β (unpublished results), probably due to autocrine or TNF-induced subeffective concentration of IFN-β, which became highly effective in the presence of TNF. Detailed investigation of the function of the various cellular proteins, the synthesis of which is enhanced in TNF-treated cells, and of the impact of these proteins on the life cycle of chlamydiae should provide further insight as to the mechanisms by which TNF inhibits chlamydial growth.

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